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The RsmA⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress $hrpN_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves

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Erwinia carotovora subsp. *carotovora* wild-type strain Ecc71 does not elicit the hypersensitive reaction (HR) in tobacco leaves. By mini-Tn*5*-Km and chemical mutagenesis we have isolated RsmA⁻ mutants of Ecc71 that produce high basal levels of pectate lyases, polygalacturonase, cellulase, and protease; they also are hypervirulent. The RsmA⁻ mutants, but not their parent strains, elicit an HR-like response in tobacco leaves. This reaction is characterized by the rapid appearance of water soaking followed by tissue collapse and necrosis. The affected areas remain limited to the region infiltrated with bacterial cells, and the symptoms closely resemble a typical HR, e.g., the reactions caused by *Pseudomonas syringae* pv. *pisi*. Moreover, low concentrations of cells of the mini-Tn*5*-Km insertion RsmA⁻ mutant, AC5070, infiltrated into tobacco leaf tissue prevent elicitation of the rapid necrosis by AC5070 or by *P. syringae* pv. *pisi*. Elicitation of the HR-like response by the mutants is not affected by the deficiency of *N*-(3-oxohexanoyl)-L-homoserine lactone, the cell density (quorum) sensing signal. Cloning and sequence analysis have disclosed that *E. carotovora* subsp. *carotovora* strain Ecc71 possesses a homolog of *E. chrysanthemi hrpN* known to encode an elicitor of the HR; the corresponding Ecc71 gene is designated $hrpN_{Ecc}$. Northern (RNA) blot data show that the level of $hrpN_{Ecc}$ mRNA is considerably higher in the RsmA⁻ mutants than in the RsmA⁺ strains. Moreover, a low copy plasmid carrying the *rsmA*⁺ allele severely reduces the level of the $hrpN_{Ecc}$ transcripts in the RsmA⁻ mutants. These constructs, like the RsmA⁺ *E. carotovora* subsp. *carotovora* strains, do not elicit the HR-like response. These data taken along with the effects of *rsmA* on exoenzyme production and pathogenicity (A. Chatterjee et al., 1995, Appl. Environ. Microbiol. 61:1959-1967) demonstrate that this global regulator gene plays a critical role in plant interaction of *E. carotovora* subsp. *carotovora*.

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Many gram-negative phytopathogenic bacteria, when infiltrated into a nonhost plant such as tobacco, cause localized necrosis, generally known as the hypersensitive reaction (HR) (Goodman and Novacky 1994). A typical HR is characterized by the rapid collapse of the leaf tissue followed by necrosis of the collapsed area. *Erwinia carotovora* subsp. *carotovora* and many other soft-rotting bacteria are unusual in that they do not elicit a typical HR when infiltrated into tobacco leaves. The inability of these bacteria to elicit the HR has been attributed to the production of pectolytic enzymes that are presumed to suppress the HR. The recent finding of Collmer and his associates that a mutant strain of *E. chrysanthemi* deficient in the synthesis of the major pectate lyase (Pel) isozymes, but not the pectolytic parent, can elicit the HR (Bauer et al. 1994) is certainly consistent with this hypothesis. In fact, both genetic and biochemical data (Bauer et al. 1995) demonstrate that *E. chrysanthemi*, like many other gram negative bacteria, possesses *hrp* genes including *hrpN*, which encodes an elicitor of the HR. These data and the results of Southern blot hybridizations of Laby and Beer (1992) support the idea that soft-rotting *Erwinia* possess *hrp* genes, but a sustained expression of *hrp* genes of these *Erwinia* species in incompatible hosts may not occur at a level required for elicitation of the HR.

We have initiated studies to clarify the genetic regulation of the production of the HR and disease symptoms by *E. carotovora* subsp. *carotovora*. We previously reported that a mini-Tn*5*-Km insertion RsmA⁻ mutant of *E. carotovora* subsp. *carotovora* is derepressed in extracellular enzyme production and it is hypervirulent (Chatterjee et al. 1995; Cui et al. 1995). A mutant of similar phenotype was also generated by chemical mutagenesis. The data presented here show that these mutants elicit responses in tobacco leaves that are similar to those in a typical HR and that they do not require the cell density sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL) to cause this reaction. Additionally, our findings disclose the presence of a homolog of the $hrpN_{Ecc}$ gene in *E. carotovora* subsp. *carotovora* strain Ecc71 and show that expression of this gene is negatively controlled by *rsmA*.

RESULTS

RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* elicit responses in tobacco leaves that resemble the HR.

Previously (Chatterjee et al. 1995; Cui et al. 1995), we have described the isolation procedure as well as some of the characteristics of *E. carotovora* subsp. *carotovora* strain AC5070, the mini-Tn*5*-Km insertion RsmA⁻ mutant (*rsm* = *r*egulator of *s*econdary *m*etabolites). Since AC5070 overproduces pectate lyases, polygalacturonases, protease, and cellulase, and is hypervirulent, it was of interest to examine the responses it could elicit in tobacco leaves, wherein wild-type *E. carotovora* subsp. *carotovora* does not cause tissue necrosis in 24 to 48 hr. As shown in Figure 1, cells of AC5070 infiltrated into tobacco leaves produced symptoms similar to those caused by *P. syringae* pv. *pisi*, known to elicit the HR. The lowest concentration of AC5070 that elicited an HR-like response was approximately 2×10^8 cells/ml. The visible symptoms, i.e., water soaking followed by tissue collapse, appeared within 24 h after the infiltration. By 24 h the inoculation sites developed necrosis, culminating in tissue desiccation. These responses, as in the typical HR, invariably remained confined to the area infiltrated with bacterial cells. Infiltration with cells of RsmA⁺ *E. carotovora* subsp. *carotovora* grown in Luria-Bertani (LB) agar did not produce visible lesions; however, after 5 to 6 days the infiltrated sites became chlorotic.

By ethyl methane sulfonate (EMS) mutagenesis of *E. carotovora* subsp. *carotovora* strain AC5006, we isolated a mutant, AC5041, that, like AC5070, overproduces pectate lyases, polygalacturonases, protease, and cellulase (Fig. 2). In addition, the mutant is hypervirulent in that it caused more severe maceration in celery petioles than the parent RsmA⁺ strain (Fig. 3). The derepressed mutant, AC5041, but not its parent strain, induced the HR-like response in tobacco leaves (data not shown).

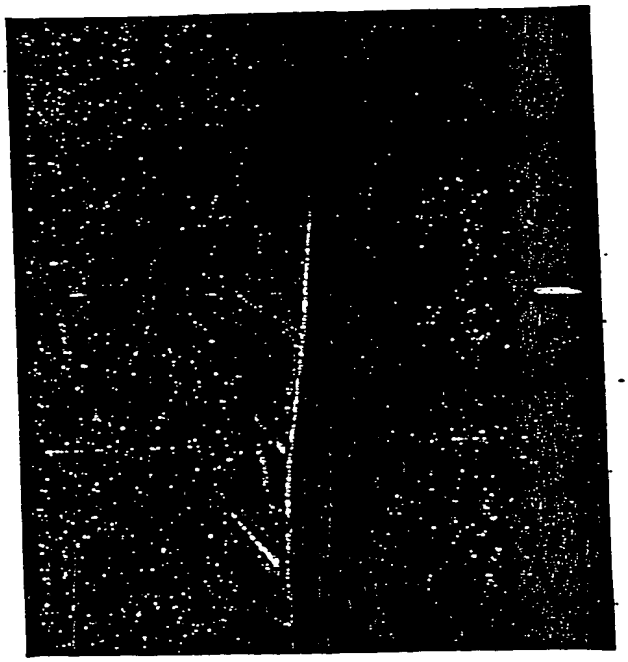

Fig. 1. Symptoms produced in tobacco leaves by *Erwinia carotovora* subsp. *carotovora* AC5047 and its RsmA⁻ mutant, AC5070. Cell suspensions containing about 2×10^8 CFU/ml were infiltrated into each leaf segment. A, AC5047; B, AC5070; C, *Pseudomonas syringae* pv. *pisi* Psp1; and D, water. Picture was taken 24 h after infiltration.

Prevention of the HR-like response.

It has been reported that *P. syringae* pv. *pisi* prevents the HR when it is preinoculated in tobacco leaves at a lower concentration (5×10^5) and later challenged with an HR-inducing concentration (5×10^6) at the same site (Novacky et al. 1973). Similarly, we have noticed that preinfiltration of tobacco leaves with AC5070 (10^5 CFU/ml) prevented the appearance of water soaking and necrosis upon reinoculation at the same

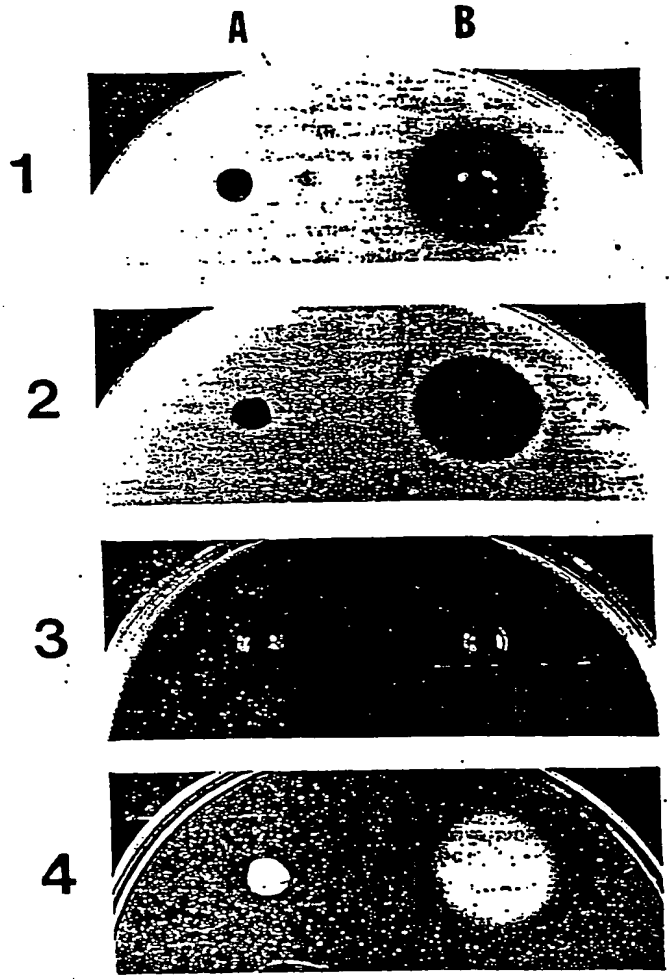

Fig. 2. Agarose plate assays for 1, pectate lyase (Pel); 2, polygalacturonase (Peh); 3, protease (Prt); and 4, cellulase (Cel) activities of *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA⁻ mutant AC5041 (B). Bacteria were grown in salts–yeast extract–glycerol medium to saturation. Culture supernatants were diluted twofold in 10 mM Tris-HCl (pH 7.0) buffer and 5 µl of the diluted samples were used for the Pel, Peh, and Cel assays. Thirty microliters of undiluted samples were used for the Prt assay.

site with AC5070 or *P. syringae* pv. *pisi* (Fig. 4). After the preinoculation, about 2×10^8 cells of AC5070 were introduced at different intervals. The ability of preinoculated cells to inhibit the HR-like response was apparent by 12 h after inoculation (data not shown), and by 24 h production of the response was completely suppressed.

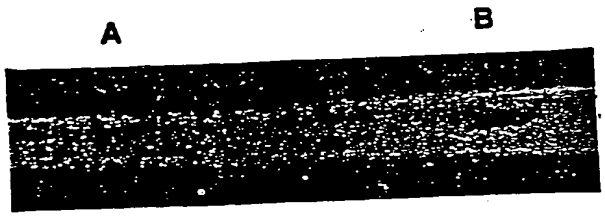

Fig. 3. Maceration of celery petioles induced by *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA⁻ mutant AC5041 (B). About 2×10^8 bacterial cells suspended in water were injected into each inoculation site. Inoculated petioles were covered with petroleum jelly and incubated in a moist chamber at 25°C for 24 h.

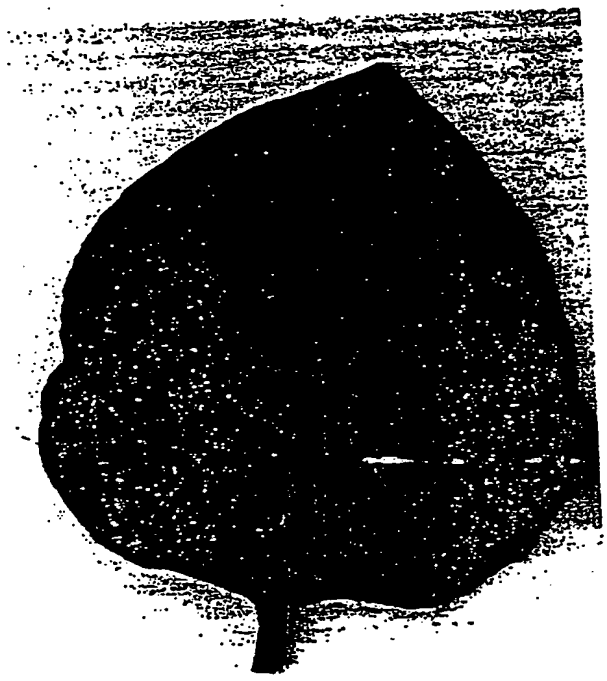

Fig. 4. Prevention of the hypersensitive response symptoms in tobacco leaf by the RsmA⁻ mutant of *Erwinia carotovora* subsp. *carotovora*, AC5070. Leaf segments were infiltrated with A, water at 0 h; B, *Pseudomonas syringae* pv. *pisi* Psp1 (5×10^8 CFU/ml) at 24 h; C, AC5070 (2×10^8 CFU/ml) at 24 h; D, AC5070 (2×10^8 CFU/ml) at 0 h; E, AC5070 (10^5 CFU/ml) at 0 h; F, AC5070 (10^5 CFU/ml) at 0 h and challenged with Psp1 (5×10^6 CFU/ml) after 24 h; G, AC5070 (10^5 CFU/ml) at 0 h and challenged with AC5070 (2×10^8 CFU/ml) after 24 h; and H, Psp1 (5×10^6 CFU/ml) at 0 hour. Leaf was photographed 48 h after infiltration.

RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* elicit the HR-like response in the absence of the cell density sensing signal, OHL.

OHL and its structural analogs are required for the expression of many phenotypes in different bacteria (Fuqua et al. 1994; Salmond et al. 1995; Swift et al. 1994). In *E. carotovora* subsp. *carotovora*, OHL controls extracellular enzyme production, pathogenicity, and production of the antibacterial antibiotic, carbapenem (Bainton et al., 1992; Chatterjee et al. 1995; Jones et al. 1993; Pirhonen et al. 1993). We had previously demonstrated that exoenzyme overproduction and pathogenicity occurred in the absence of OHL in the RsmA⁻ mutant, AC5070 (Chatterjee et al. 1995). To find out if the mutants could elicit the HR-like response in the absence of this cell density sensing signal, we examined the responses induced by OHL-deficient derivatives of the RsmA⁻ strains. We made the EMS-induced RsmA⁻ mutant OHL deficient by replacing *ohlI*⁺ (previously designated as *hslI*⁺) allele required for OHL biosynthesis, with *ohlI*-MudI by marker exchange, as we had done with AC5070 (Chatterjee et al. 1995). AC5090 and AC5093, the derivatives of AC5070 and AC5041, respectively, do not produce OHL, as indicated by the Lux bioassay (Chatterjee et al. 1995; data not shown). Figure 5 shows that AC5090 and AC5093 elicited reactions in tobacco leaves that were very similar to those produced by the parent strains as well as by *P. syringae* pv. *pisi*.

The RsmA⁻ mutants overexpress $hrpN_{Ecc}$, a locus presumed to specify an elicitor of the HR.

Recent studies by S. V. Beer, A. Collmer, and their associates demonstrated that *hrpN* genes of *E. amylovora* and *E. chrysanthemi* encode elicitors of the HR and raised the possi-

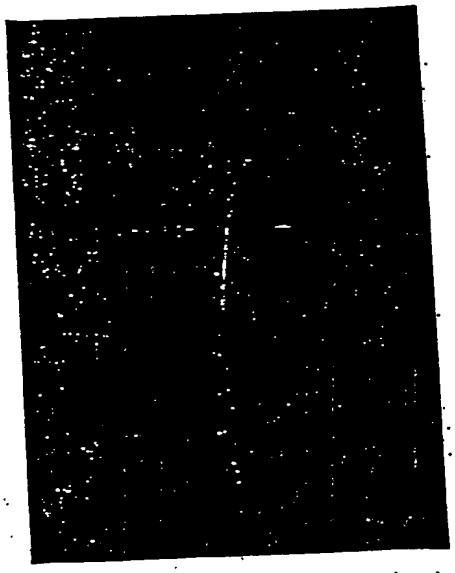

Fig. 5. Elicitation of the hypersensitive-like response in tobacco leaves by RsmA⁻ mutants of *Erwinia carotovora* subsp. *carotovora* and their OhlI⁻ derivatives. Leaf segments were infiltrated with 2×10^8 CFU/ml of bacterial cells. A, water; B, AC5093 (RsmA⁻, OhlI⁻); C, AC5090 (RsmA⁻, OhlI⁻); D, *Pseudomonas syringae* pv. *pisi* Psp1; E, AC5041 (RsmA⁻, OhlI⁺); and F, AC5070 (RsmA⁻, OhlI⁻). Picture was taken 24 h after infiltration.

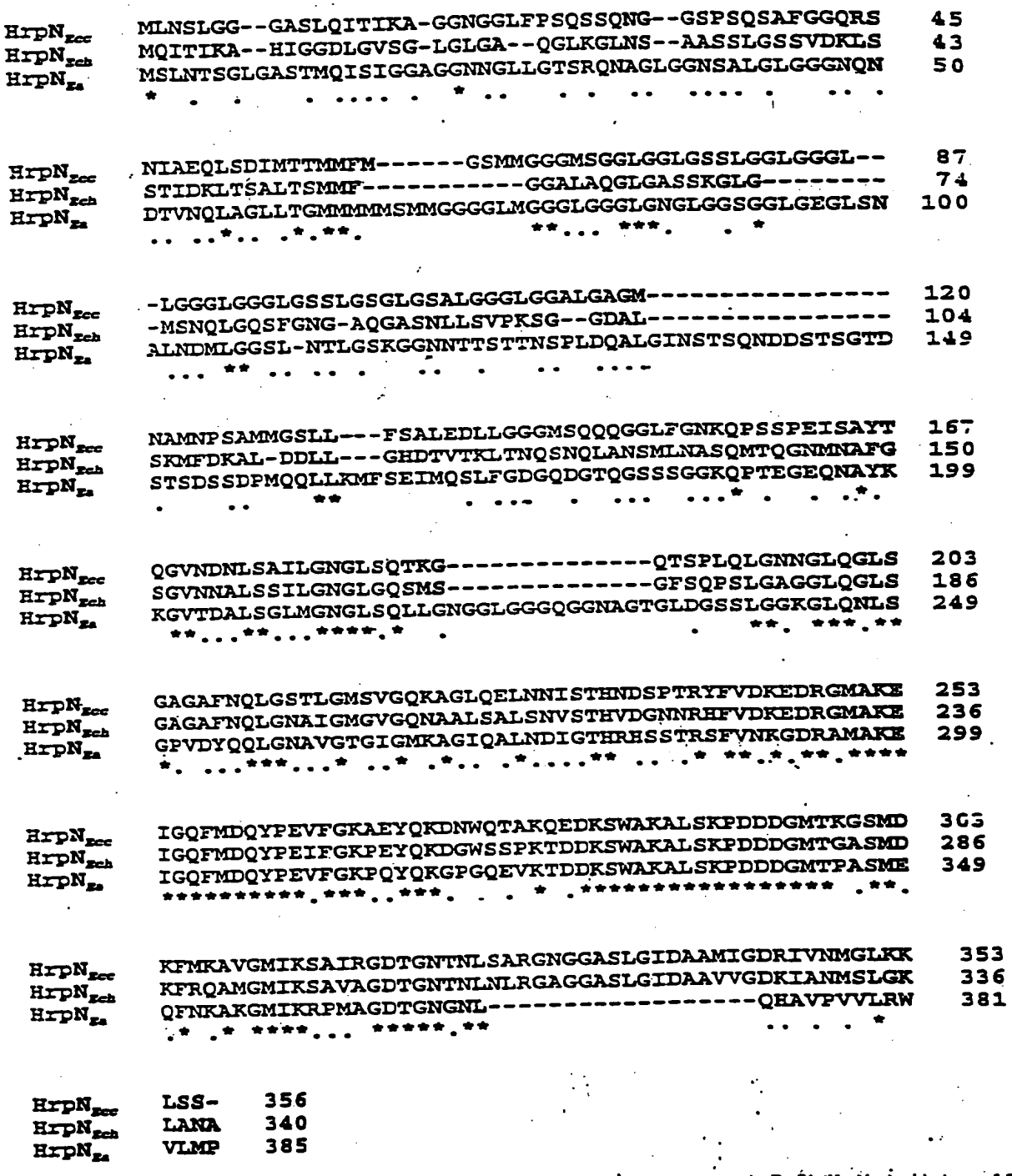

Fig. 6. Alignment of deduced amino acid sequence of *hrpN*$_{Ecc}$ of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 (HrpN$_{Ecc}$) with those of *E. chrysanthemi* EC16 (HrpN$_{Ech}$) and *E. amylovora* Ea321 (HrpN$_{Ea}$). Asterisks indicate identical amino acids; single dots indicate conservative substitutions. Numbers at R right indicate amino acid positions in each protein.

bility that *hrp* genes including *hrpN* may also occur in other *Erwinia* species (Bauer et al. 1994; Bauer et al. 1995; Laby and Beer 1992; Wei et al. 1992). Indeed, Southern blot hybridization under moderate stringency conditions with *hrpN* DNA of *E. chrysanthemi* (EC16) (Bauer et al. 1995) as the probe disclosed the presence of *hrpN* sequences in *E. carotovora* subsp. *carotovora* strain Ecc71 (data not shown). Subsequently, by screening a library of Ecc71 with the *hrpN* DNA of *E. chrysanthemi*, several clones p ssessing homologous DNA were identified; the corresponding Ecc71 sequences are tentatively designated as $hrpN_{Ecc}$. Sequence analysis of the DNA segment that specifically hybridized with the *hrpN* DNA of *E. chrysanthemi* revealed an 1,068-bp open reading frame whose predicted product has 72.1% similarity and 53.4% identity with the deduced product of *hrpN* of *E. chrysanthemi*, and 66.6% similarity and 50.8% identity with the predicted product of *hrpN* of *E. amylovora* (Fig. 6).

Northern (RNA) blot analysis was performed with total RNA preparations from the wild-type strain Ecc71, the $RsmA^-$ mutants, AC5041 and AC5070, and their $RsmA^+$ parents to ascertain if $hrpN_{Ecc}$ expression is derepressed in the $RsmA^-$ strains. Bacteria were grown in SYG medium at 28°C to a Klett value of approximately 200 and used for total RNA isolation. A 700-bp *Acc*I-*Sma*I internal fragment of the $hrpN_{Ecc}$ was used as the probe. The data (Fig. 7) revealed the presence of 1100-base transcripts in AC5070 and AC5041. By contrast, these transcripts were not detected with $RsmA^+$ strains 71, AC5006 and AC5047. We should note that somewhat higher levels of $hrpN_{Ecc}$ transcripts were present in the mini-Tn*5*-Km insertion mutant (AC5070) than in the EMS-induced mutant (AC5041). We do not yet know the reason for this difference. It is possible that AC5041 produces a defective RsmA with a leaky activity, whereas the mini-Tn*5*-Km insertion mutant does not produce a functional RsmA. It is, however, clear that $hrpN_{Ecc}$ transcripts are substantially higher in AC5041 than in its $RsmA^+$ parent, AC5006.

The *rsmA*$^+$ allele suppresses elicitation of the HR-like response and expression of $hrpN_{Ecc}$

We have previously described the cloning and characterization of the *rsmA* gene of *E. carotovora* subsp. *carotovora* strain Ecc71 (Chatterjee et al. 1995; Cui et al. 1995). A low-copy plasmid carrying this gene causes a severe attenuation of pathogenicity and suppresses extracellular enzyme production in *E. carotovora* subsp. *carotovora* and *E. c.* subsp. *atroseptica*; represses pathogenicity, exopolysaccharide production, flagellum production and motility, protease production, and elicitation of the HR by *E. amylovora*; and suppresses extracellular enzyme and antibiotic production by *E. carotovora* subsp. *betavasculorum* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). In light of the large array of effects on phenotypes by *rsmA*, including induction of the HR by *E. amylovora*, it was deemed worthwhile to examine the effects of the *rsmA*$^+$ DNA on elicitation of the HR-like response by the mutants. The plasmids pCL1920 and pAKC880 were transformed into AC5041 and AC5070 and the constructs were tested for induction of the HR-like response. Figure 8 shows that AC5041 and AC5070 carrying the cloning vector, pCL1920, elicited reactions in tobacco leaves similar to those caused by *P. syringae* pv. *pisi*. By contrast, there was no visible reaction in the leaf segment infiltrated with AC5041 or AC5070 carrying the $RsmA^+$ plasmid, pAKC880. These results indicate that multiple copies of *rsmA* suppress elicitation of the HR-like response in tobacco leaves by AC5041 and AC5070.

Northern analysis was conducted to determine the effect of RsmA plasmid on $hrpN_{Ecc}$ transcription. The data (Fig. 9) show that high levels of $hrpN_{Ecc}$ transcripts were present in cells of AC5041 and AC5070 containing the cloning vector, pCL1920, but the transcripts were not detected in cells carrying the *rsmA* plasmid, pAKC880.

DISCUSSION

We previously reported that extracellular enzyme production as well as virulence are negatively regulated by *rsmA* in *E. carotovora* subsp. *carotovora* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). For example, the inactivation of *rsmA* by a transposon resulted in overproduction of extracellular enzymes and hypervirulence. Moreover, unlike its parent, the $RsmA^-$ mutant did not require the cell density sensing signal, OHL, for pathogenesis or extracellular enzyme production. In this report, we have shown that this $RsmA^-$ mutant and an EMS-induced mutant of a similar phenotype elicited the HR-like response in tobacco leaves, and that the elicitation of this reaction was also not dependent upon OHL. Although we do not yet have direct evidence that the mutations in AC5041 and AC5070 are in the same gene, these strains possess similar phenotypes; e.g., they overproduce extracellular enzymes, they are hypervirulent, and OHL deficiency does not affect the expression of these traits. Moreover, the plasmid carrying *rsmA*$^+$ DNA suppresses extracellular enzyme production, pathogenicity, and the elicitation

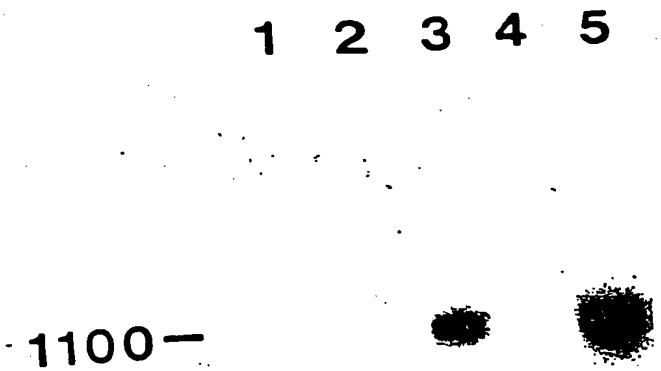

Fig. 7. Northern (RNA) blot analysis of $hrpN_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* strains. Each lane contained 20 µg of total RNA. Position of 1100-base transcript is indicated. Lane 1, Ecc71 (wild-type parent, $RsmA^+$); lane 2, AC5006 ($RsmA^+$); lane 3, AC5041 ($RsmA^-$); lane 4, AC5047 ($RsmA^+$); lane 5. AC5070 ($RsmA^-$).

of the HR-like response by the mutants. Also, both the mutants express $hrpN_{Ecc}$ constitutively, although the transcript level is somewhat higher in AC5070 than in AC5041. As thes mutants have similar phenotypes, we tentatively classified them as RsmA$^-$.

The following lines of evidence strongly suggest that the mutants elicited a typical HR (Goodman and Novacky 1994): (i) the reaction was characterized by a rapid physiological activity (i.e., water movement or water soaking), tissue collapse followed by cell death (necrosis); (ii) the affected areas were limited to the region infiltrated with bacterial cells; (iii) these symptoms were indistinguishable from the symptoms developed by *P. syringae* pv. *pisi*, a bacterium known to elicit the typical HR in tobacco leaves; (iv) the response elicited by AC5070 was preventable upon previous infiltration of a low concentration of AC5070 cells and, similarly, prior inoculations with AC5070 cells prevented elicitation of the HR by *P. syringae* pv. *pisi*; and (v) while AC5070 and AC5041, their parent strains, and the wild-type strain possess $hrpN_{Ecc}$ sequences (data not shown), the expression of $hrpN_{Ecc}$ is derepressed only in the mutants, presumably leading to the production of high levels of a putative elicitor of the HR (see below).

Our observations support the idea that AC5070 and AC5041 produce an elicitor that triggers the HR-like response in tobacco leaves. We attribute the manifestation of this response with the mutants, but not with the parents, t the ability of the former to produce high constitutive levels of $HrpN_{Ecc}$, an exoenzyme, or both. With regard to the possible role of exoenzymes, it is perhaps significant that pectinases are known to generate elicitors of plant defense responses (Davis et al. 1984; Davis and Ausubel 1989; Keen 1992). Furthermore, Palva et al. (1993) have documented the activation of chitinases and glucanases in tobacco by exoenzyme-producing strains of *E. carotovora* subsp. *carotovora* but not by mutants deficient in exoenzyme production. Therefore, one could argue that pectinase overproduction by the RsmA$^-$ mutants may induce defense reactions that could culminate in an HR-like response. The inability of the wild-type RsmA$^+$ *E. carotovora* subsp. *carotovora* strain Ecc71 to elicit this response could be attributed to the lack of extracellular enzyme production in a nonhost tissue, i.e., in a tobacco leaf. However, the hypothesis implicating pectolytic enzymes as elicitors of the HR is difficult to reconcile with the finding of Bauer et al. (1994) that only those mutants of *E. chrysanthemi* that are deficient in major pectate lyases can elicit the HR.

In light of that finding and for the following reasons, we favor the hypothesis that induction of the HR-like response by the mutants may be due to the derepression of a gene encoding an elicitor, such as $HrpN_{Ech}$ or $HrpN_{Ecr}$. Collmer and asso-

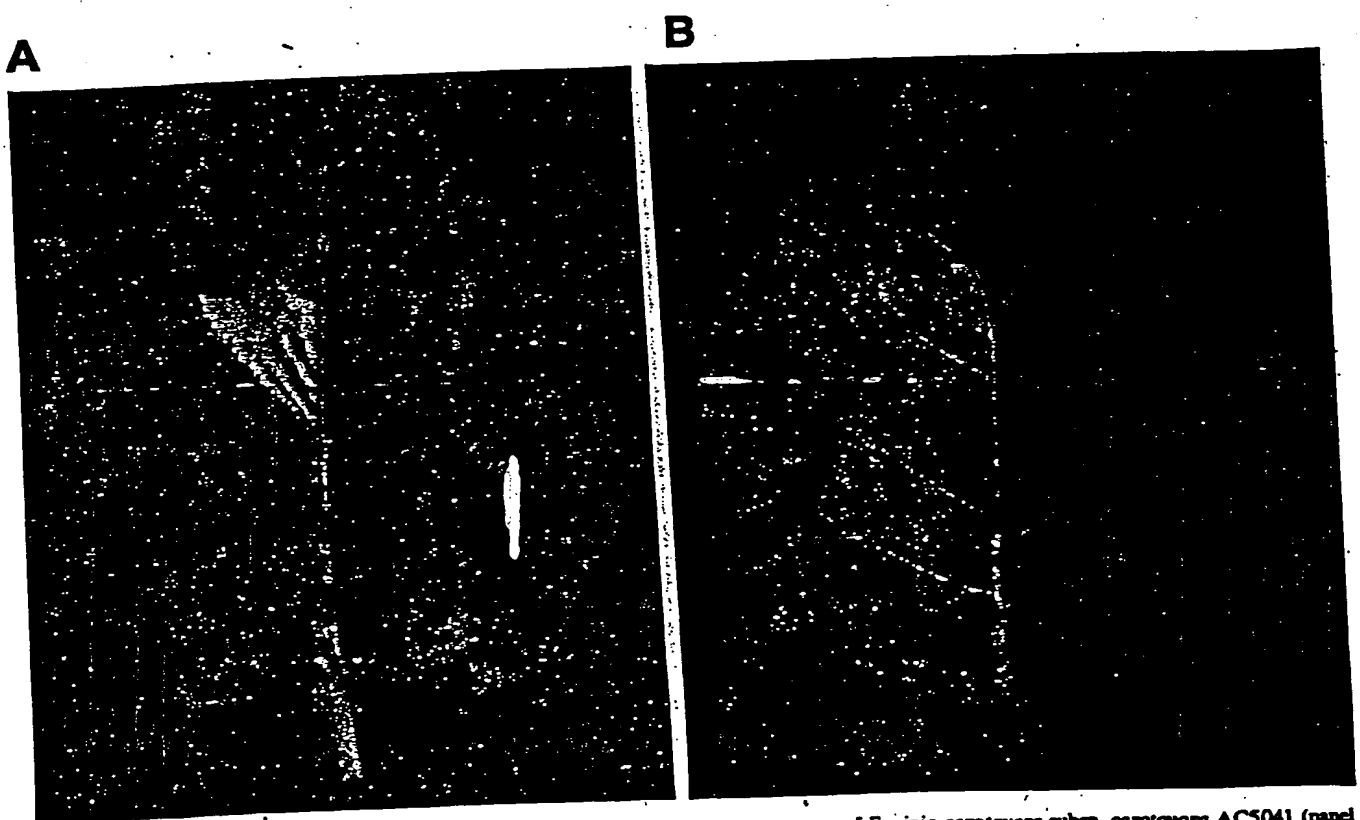

Fig. 8. Elicitation of an hypersensitive-like response in tobacco leaves by the RsmA$^-$ mutants of *Erwinia carotovora* subsp. *carotovora* AC5041 (panel A) and AC5070 (panel B) carrying the *rsmA*$^+$ plasmid, pAKC880, or the cloning vector, pCL1920. Bacterial suspensions containing about 2×10^8 CFU/ml were infiltrated into each leaf segment. Panel A: A, *Pseudomonas syringae* pv. *pisi* Psp1; B, AC5041 carrying pAKC880; C, water; D, AC5041 carrying pCL1920. panel B: A, Psp1; B, AC5070 carrying pAKC880; C, water; D, AC5070 carrying pCL1920. Picture was taken 24 h after infiltration.

ciates (Bauer et al. 1994; Bauer et al. 1995) have discovered a gene specifying an elicitor of the HR in the soft-rotting bacterium *E. chrysanthemi*. The deduced sequence of $HrpN_{Ecc}$ presented here document the occurrence of a homolog of *E. chrysanthemi hrpN* in *E. carotovora* subsp. *carotovora* strain Ecc71. We have found that the mini-Tn*5*-Km induced $RsmA^-$ mutant as well as the EMS-induced derepressed mutant possess a substantial level of an approximately 1100-base transcript that specifically hybridizes with the $hrpN_{Ecc}$ DNA. By contrast, this transcript is barely detectable in the $RsmA^+$ strains. Moreover, the introduction of the $rsmA^+$ allele into the mutants severely reduces the levels of this transcript and concomitantly abolishes the ability to elicit the HR-like response. These observations indicate that transcription of $hrpN_{Ecc}$ is derepressed in the mutants, and that this derepression is due to the inactivation of *rsmA*. At the moment, since the genes for pectolytic enzymes and $hrpN_{Ecc}$ are both derepressed in the $RsmA^-$ mutants, we have to entertain the possibility that the pectolytic enzymes could also contribute to the hypersensitive necrosis of tobacco leaf tissue. Genetic and biochemical studies have been initiated to determine if $hrpN_{Ecc}$ and its putative product are solely responsible for the elicitation of the HR and to clarify the ramifications of $hrpN_{Ecc}$ regulation in compatible and incompatible interactions of *E. carotovora* subsp. *carotovora*.

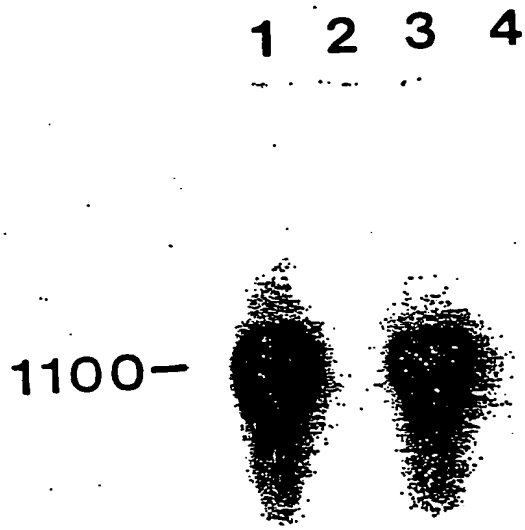

Fig. 9. Northern (RNA) blot analysis of $hrpN_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* $RsmA^-$ mutants AC5041 and AC5070 carrying the $rsmA^+$ plasmid, pAKC880, or the cloning vector, pCL1920. Each lane contained 20 µg of total RNA. The position of 1100-base transcript is indicated. Lane 1, AC5070 carrying pCL1920; lane 2, AC5070 carrying pAKC880; lane 3, AC5041 carrying pCL1920; lane 4, AC5041 carrying pAKC880.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids are described in Table 1. *E. carotovora* subsp. *carotovora* strains were routinely grown in LB and *P. syringae* pv. *pisi* on King's B (King et al. 1954) agar media at 28°C. Minimal salts plus sucrose (0.2%) agar, nutrient gelatin (NG) agar, polygalacturonate-yeast extract agar (PYA) and salts-yeast extract-glycerol (SYG) media have been described previously (Barras et al. 1987; Chatterjee 1980; Murata et al. 1991). When required, antibiotics were added at the indicated concentrations in micrograms per milliliter: spectinomycin (Spc), 50; tetracycline (Tc), 10; Ampicillin (Ap), 50 and Kanamycin (Km), 50. The composition of agarose media for semiquantitative assays of enzymatic activities has been described in Chatterjee et al. (1995).

Enzyme assays.

The preparation of enzyme samples for assays as well as the assay procedures were described previously (Murata et al. 1991; Chatterjee et al. 1995). The volumes of enzyme samples used in the assays are indicated in the figure legends.

Bioluminescence assay for OHL.

The procedure described by Chatterjee et al. (1995) was followed.

Recombinant DNA techniques.

Standard procedures were followed in DNA isolation, transformation and electroporation of bacteria, restriction digests, gel electrophoresis, DNA ligation, and Southern blot analysis (Sambrook et al. 1989). Restriction and modifying enzymes were obtained from Promega Biotech (Madison, WI).

Isolation of $RsmA^-$ mutants.

The procedure used for the isolation of AC5070 by mini-Tn*5*-Km has been described (Chatterjee et al. 1995). AC5041 was isolated by EMS mutagenesis of AC5006. Mutagenesis was carried out according to the protocol of Miller (1972). The bacterial cells were incubated with EMS for a period that yielded less than 5% survival. The putative $RsmA^-$ mutants were identified by their ability to overproduce protease, cellulase, and pectolytic enzymes in agar plate assays (Chatterjee et al. 1995).

Inactivation of the *ohl* locus by MudI mutagenesis.

The plasmid, pAKC852, carrying the 9.7-kb ohl^+ DNA of *E. carotovora* subsp. *carotovora* strain Ecc71 was mutagenized with MudI1734 following the procedure of Castilho et al. (1984). Briefly, pAKC852 was transformed into the lysogenic *Escherichia coli* strain POI1734. The strain carrying the Ohl^+ plasmid was heat-induced to lyse. The lysate was used to transduce *E. coli* M8820, and the Tc^rKm^r transductants were screened for OHL production by means of the plate assay procedure described in Chatterjee et al. (1995). Plasmids were isolated from M8820 colonies that could no longer activate the *lux* operons in pHV200L.

Construction of bacterial strains by marker exchange.

The construction of AC5090, the Ohl^- derivative of AC5070, has been described (Chatterjee et al. 1995). To isolate AC5093, the Ohl^- mutant of AC5041, the plasmid (pAKC863) carrying inactivated *ohlI*-MudI was transferred into AC5041 by means of the helper plasmid, pRK2013.

Transconjugants were selected on minimal salts plus sucrose agar supplemented with Km. Col nies that were Km^{r}Tc^s were tested for the Ohl phenotype. AC5093 was selected for further studies.

Plant tissue macerati n.

The celery petiole assay was previously described (Murata et al. 1991). The extent of tissue maceration was estimated visually.

Infiltration of tobacco leaves.

Erwinia species were grown on LB agar and *P. syringae* pv. *pisi* was grown on King's B agar overnight at 28°C and cells were resuspended in water. Strains carrying plasmids were grown on LB agar containing spectinomycin and cells suspended in a 50 μg/ml spectinomycin solution in water. Young, fully expanded third and fourth leaves of about 8-week-old *Nicotiana tabacum* L. cv. Samsun were infiltrated with bacterial suspensions. Inoculated plants were incubated in a growth chamber at 27°C with a 14/10 h daylight regime and visually monitored for reactions. For testing the prevention of the HR-like response, cells of AC5070 (10^5 CFU/ml) were infiltrated into tobacco leaves. The preinoculated areas were reinoculated with 2×10^3 CFU of AC5070 per ml or 5×10^6 CFU of *P. syringae* pv. *pisi* Psp1 per ml at desired intervals.

Cloning of $hrpN_{Ecc}$ DNA and nucleotide sequence analysis.

The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *Cla*I fragment of *hrpN* of *E. chrysanthemi* (Bauer et al. 1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

Unidirectional 5′ to 3′ deletions of pAKC924 were made and the overlapping deletions differing in size by approximately 200 bp were used for sequence analysis with the Sequenase System II (U.S. Biochemicals, Cleveland, OH). In addition, we used oligonucleotide primers to verify and complete the sequence of $hrpN_{Ecc}$ with pAKC923 and pAKC924 DNAs as templates. Alignment of protein sequences was performed using the Genetics Computer Group, Inc. (Madison, WI) software program at the DNA Core facility on the University of Missouri-Columbia campus and the PC/GENE program (IntelliGenetics, Inc., Mountain View, CA). The sequence of $hrpN_{Ecc}$ has been deposited at GenBank and has been assigned accession number L78834.

Northern blot analysis.

Bacterial cultures were grown to a value of approximately 200 Klett units at 28°C in SYG medium with or without

Table 1. Bacterial strains and plasmids

Bacteria	Relevant characteristics[a]	Reference or source
Erwinia carotovora subsp. *carotovora*		
71	Wild type	Zink et al. 1984
AC5006	Lac$^-$ mutant of 71	Murata et al. 1991
AC5041	RsmA$^-$, EMS mutant of AC5006	This study
AC5047	Nal^r derivative of AC5006	Chatterjee et al. 1995
AC5070	RsmA$^-$, mini-Tn*5*-Km mutant of AC5047, Km^r, Nal^r	Chatterjee et al. 1995
AC5090	Ohl$^-$ derivative of AC5070, RsmA$^-$, Km^r, Spc^r	Chatterjee et al. 1995
AC5093	Ohl$^-$ derivative of AC5041, RsmA$^-$, Km^r	This study
Pseudomonas syringae pv. *pisi*		
Psp1	Wild type	A. J. Novacky
Escherichia coli		
DH5α	*ϕ80lacZ ΔM15, Δ(lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1*	BRL, Frederick, MD
HB101	*proA1 lacY hsdS20(rB$^-$ mB$^-$), recA56 rpsL20*	Zink et al. 1984
M8820	*Δ(proAB-argF-lacPOZYA)recA$^+$*	Castilho et al. 1984
POI1734	MudI1734::ara(Mu cts), *Δ(proAB-argF-lacIPOZYA)*	Castilho et al. 1984
VJS533	*ara Δ(lac-proAB) rpsL ϕ80lacZ ΔM15 recA56*	Gray and Greenberg 1992
Plasmids		
pAKC852	Ohll$^+$, Tc^r	Chatterjee et al. 1995
pAKC863	Derived from pAKC852, *ohll*::MudI, Km^r, Tc^r	This study
pAKC880	RsmA$^+$, Spc^r	Cui et al. 1995
pAKC921	pLARF5 containing $hrpN_{Ecc}$ from genomic library of Ecc71, Tc^r	This study
pAKC922	pLARF5 containing $hrpN_{Ecc}$ from genomic library of Ecc71, Tc^r	This study
pAKC923	4.0-kb *Eco*RI fragment of pAKC921 containing $hrpN_{Ecc}$ cloned into pSK$^+$, Ap^r	This study
pAKC924	1.4-kb *Eco*RI fragment of pAKC922 containing $hrpN_{Ecc}$ cloned into pSK$^+$, Ap^r	This study
pCL1920	Spc^r	Lerner and Inouye 1990
pCPP2172	$hrpN_{Ecc}$, Ap^r	Bauer et al. 1995
pLARF5	Tc^r	Keen et al. 1988
pRK415	Tc^r	Keen et al. 1988
pRK2013	Mob$^+$, Tra$^+$, Km^r	Figurski and Helinski 1979
pBluescript SK+	Ap^r	Stratagene, La Jolla, CA
pHV200	8.8-kb *lux* DNA in pBR322, Ap^r	Gray and Greenberg 1992
pHV200I	Frameshift mutation of luxI in pHV200, Ap^r	Pearson et al. 1994

[a] Uncommon abbreviations: EMS = ethyl methane sulfonate; Ohl = *N*-(3-oxohexanoyl)-L-homoserine lactone, designated as Hsl in our previous publications; *rsmA* = regulator of secondary metabolites; $hrpN_{Ecc}$ = *E. carotovora* subsp. *carotovora* DNA fragment carrying a $hrpN_{Ech}$ homolog (Bauer et al. 1995).

spectinomycin. The procedures for RNA isolation and Northern blot analysis described in Chatterjee et al. (1991) and Liu et al. (1993) were followed. A 0.7-kb *AccI-SmaI* internal fragment of $hrpN_{Ecc}$ was used as the probe.

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